EFFECT OF PLANT HORMONES ON ULVA[1]

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Föyn (1934a) in his early attempts to grow *Ulva lactuca* found that *Ulva,* like *Cladophora suhriana* (Föyn, 1934b), grows poorly in sea water enriched with nitrates and phosphates (Schreiber, 1927) and that the addition of soil extract to Schreiber's medium is necessary to obtain normal growth and the entire life-cycle. This medium ("Erdschreiber") later became the standard medium for growing marine flagellates in bacterized cultures (Gross, 1937; Parke, 1949).

Kylin (1941) employed *Ulva lactuca* to analyze the biological activity of different samples of sea water: he found that sea water at 70 meters depth is inadequate to support normal growth and that addition of nitrates, phosphates and trace metals made it suitable for the germination of the zoospores of *Ulva* and elicited as rapid growth of the germlings to the stage of 15–20 cells as did the "Erdschreiber."

Levring (1946), employing the same technique and test organism, formulated a synthetic sea water which, similarly enriched, allowed normal development of the germlings of *Ulva,* thus confirming, with a chemically defined medium, Kylin's conclusion. Levring's medium was the starting point for the formulation of several synthetic marine media which do not precipitate and are suitable for the cultivation of a number of marine and brackish algal flagellates in bacteria-free culture (Provasoli, McLaughlin and Droop, 1957).

Since Föyn, Kylin and Levring worked with bacterized cultures, I wondered if *Ulva,* when bacteria-free, would grow in mineral media or if it would require organic factors. Many other algae which, like *Ulva,* were previously cultured in Erdschreiber + bacteria, require, besides nitrates and phosphates, growth factors and trace metals when cultured in synthetic media without bacteria (Provasoli and Pintner, 1953; Sweeney, 1954; Lewin, 1954; Droop, 1955a, 1955b, 1957; Provasoli, 1957).

In exploratory attempts to grow *Ulva* in bacteria-free culture, I failed to obtain a typical foliaceous thallus but in trying to obtain it, I found that *Ulva* germlings respond to plant hormones.

Material and Methods

Bacteria-free cultures of *Ulva* were obtained by placing pieces of thallus on the surface of agar media containing various concentrations of an antibiotic mixture (1 ml. of the concentrated antibiotic solution contains: K penicillin G 12,000 units; chloramphenicol 50 μg.; polymyxin B 50 μg.; neomycin 60 μg.).

I recognized from the beginning the necessity of employing thalli free from epiphytic organisms: the pieces of thallus were selected and inspected under the dissecting microscope and, as an additional precaution, were cleaned by brushing

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the two surfaces with a thick, soft water-color brush. Even so, the epiphytes could not always be eliminated and several bacteria-free cultures of *Ulva* were infected with small diatoms (mainly *Nitzschia*).

The concentrated antibiotic mixture was sterilized by filtration through a glass filter, and 0.1-, 0.2-, 0.3-ml. aliquots were dispensed on the bottom of sterile Petri dishes; 20 ml. of sterile 1.4% agar media, kept at 45° C., were added and thoroughly mixed with the antibiotic by twirling. The pieces of *Ulva* thallus (5-mm. squares) were left on this agar for 7 days, removed aseptically with a spatula, placed in depression slides containing sterile media, cut in several narrow strips with an iridectomy scalpel, and transferred to liquid media. The pieces treated with the lower concentrations of antibiotics (0.1–0.2 ml. in 20 ml. of agar media) were infected with either bacteria, a pink yeast, or diatoms. All the pieces treated with 0.3 ml. of antibiotic mixture (final concentration of antibiotics per ml. of agar medium: K penicillin 200 units, and 1 μg. each of chloramphenicol, neomycin, and polymyxin) were bacteria-free but about ⅓ were infected by diatoms.

Several liquid media were tried: the most successful were ASW III and ASW 8 (Table I); both are enriched sea water media similar to, but richer than, Erdschreiber. ASW III (richer in organics) was employed in the early experiments; later I employed ASW 8 which allows better growth.

The cultures are carried in screw-cap tubes (125 × 20 mm.) with 10 ml. of medium; to avoid chemical contamination we employ plastic caps without liners. At first, *Ulva* was grown in continuous light (200 foot-candles; fluorescent tubes) at 18–20° C., later in alternate light (16 hours) and darkness (8 hours).

The sample of gibberellins was kindly supplied by Dr. Nickell, of Chas. Pfizer & Co., and the one of kinetin bought from the California Foundation for Biochemical Research.

Results

The purified strips of thallus, when transferred to liquid media, began to produce thin filamentous germlings from their surface and looked like pincushions. That many zoospores were also set free was evident from the many small germlings that covered the walls of the test tubes.

The germlings arising from the thallus were round, thin, solid, and never became more than 2–4 mm. long; after two months they bleached, leaving a few intensely green spots which dotted their surface at random. The germlings on the walls of the tubes in certain media behaved similarly and produced a number of rhizoids, many of them colorless; in other media, the germlings developed only rhizoids and looked like stellate colonies or like an elongated root system in miniature. Pieces of bleached stubby germlings, or the stellate rhizoidal colonies, when transferred to new media, produce new germlings from the islands of intensely green cells which are scattered among the bleached tissues; these green cells remain dormant and viable for a year (longest time tried) in the old medium.

The germlings of the second generation underwent a similar cycle, they grew a few millimeters and later bleached partially; no zoospores were produced by these germlings. Serial transfers are carried out by removing the old germlings aseptically from the culture tubes, cutting them in pieces and inoculating the pieces in different media.

Föyn obtained normal development of the thallus, production of zoospores and gametes of *Ulva*, in bacterized cultures grown in Erdschreiber. ASW III, an Erdschreiber enriched with vitamins, liver extract, and carbon sources, allowed only the formation of germlings; the typical thallus was never obtained in bacteria-free cultures in this medium. The beginning of a thallus (the formation of two short, thick "rabbit ears" or a fan-like curly, thick, small thallus) was obtained in

TABLE I

Media for Ulva

	Föyn's Erdschreiber	ASW III	ASW 8
Sea water	100 ml.	100 ml.	80 ml.
H_2O			20 ml.
$NaNO_3$	10 mg.		30 mg.
KNO_3		20 mg.	
$Na_2HPO_4 \cdot 12\ H_2O$	2 mg.		
K_2HPO_4		2 mg.	
Na_2 glycerophosphate			3 mg.
Mn (as Cl)		0.04 mg.	
Fe (as Cl)		0.01 mg.	0.05 mg.
P II metals*			3 ml.
Vitamin mix No. 8**		0.1 ml.	
Vitamin mix S. 3***			0.5 ml.
B_{12}			0.01 μg.
Liver 1:20†		1. mg.	
Soil extract	5 ml.	4 ml.	
Na H glutamate		50 mg.	
Glycine		50 mg.	
Tris (hydroxymethyl) amino-methane††		100 mg.	100 mg.
pH	8.0	7.5	8.0

* One ml. of P II metal contains: ethylenediamine tetraacetic acid, 1 mg.; Fe (as Cl) 0.01 mg.; B (as H_3BO_3) 0.2 mg.; Mn (as Cl) 0.04 mg.; Zn (as Cl) 0.005 mg.; Co (as Cl) 0.001 mg.

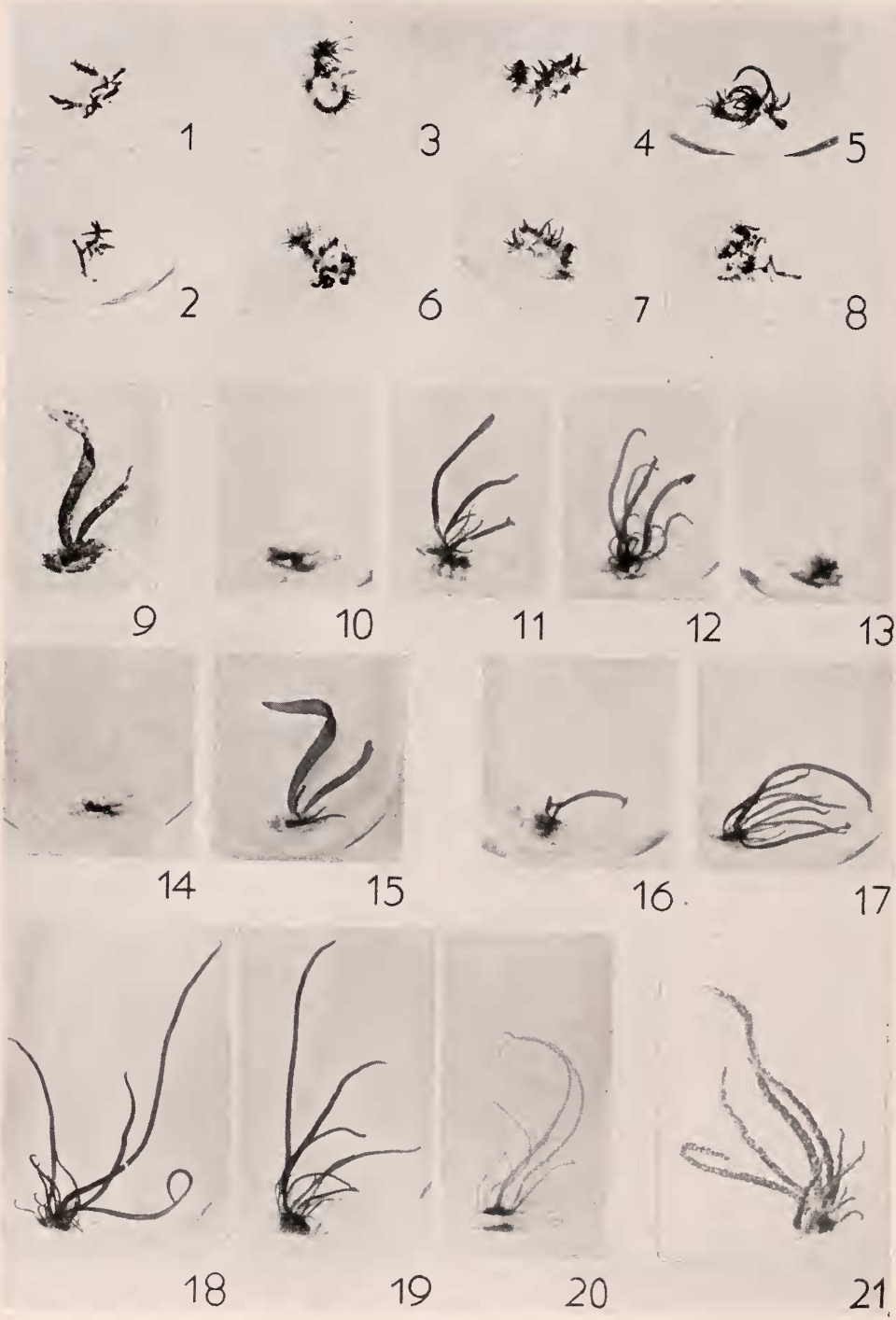
** One ml. of Vitamin mix No. 8 contains: thiamine HCl, 0.2 mg.; nicotinic acid, 0.1 mg.; putrescine 2 HCl, 0.04 mg.; Ca pantothenate, 0.1 mg.; riboflavin, 5.0 μg.; pyridoxine 2 HCl, 0.04 mg.; pyridoxamine 2 HCl, 0.02 mg.; para-aminobenzoic acid, 0.01 mg.; biotin, 0.5 μg.; choline H_2 citrate, 0.5 mg.; inositol, 1.0 mg.; thymine, 0.8 mg.; orotic acid, 0.26 mg.; B_{12}, 0.05 μg.; folinic acid, 0.2 μg.; folic acid, 2.5 μg.

*** One ml. of Vitamin mix S. 3 contains: thiamine HCl, 0.05 mg.; nicotinic acid, 0.01 mg.; Ca pantothenate, 0.01 mg.; *p*.-aminobenzoic acid, 1.0 μg.; biotin, 0.1 μg.; inositol, 0.5 mg.; folic acid 0.2 μg.; thymine, 0.3 mg.

† Nutritional Biochemical Corporation, Cleveland, Ohio, U. S. A.

†† "Sigma 7–9 biochemical buffer," Sigma Chemical Co., St. Louis, Missouri, U. S. A.

two of the tubes which were infected with a pink yeast or a diatom. However, re-infection of the axenic cultures of *Ulva* germlings with clonal cultures of the yeast or diatom failed to produce a thallus. Disappointed by the failure to obtain normal thalli, I re-examined Föyn's cultural methods and noted that he had found (1955) that only the northern European variety of *Ulva* can be grown in continuous light, that the southern variety (which he proposed to call *Ulva thureti*) bleached if grown in continuous light and that a normal thallus was formed only with a daily period of 6 or more hours of darkness. From then on, all cultures had an 8-hour



FIGURES 1–21.

dark period and 16 hours light but no thallus formed and the germlings bleached as before.

The repeated failure to obtain normal morphogenesis of the thallus in bacteria-free culture, in media similar to Erdschreiber, suggested that the failure was due to a lack of morphogenetic regulators; some of them, like indolacetic acid and gibberellin, can be produced by microorganisms. I tried various concentrations and combinations of adenine, indolacetic acid (IAA) and kinetin which influence growth and differentiation in higher plants.

In the first experiments we found that adenine by itself favored the production of more germlings from the dormant green cells and induced longer filaments; the best concentration was 3 mg.% (6 mg.% is inhibitory).

In the presence of 1 μg.% kinetin, indolacetic acid induced a large number of germlings whose length tended to increase proportionally with the concentration of IAA: 5 μg.% was the best. When 3 mg.% adenine was superadded, the most effective concentration of IAA was 10μg.%; 30μg.% inhibited the length and number of germlings.

In the presence of 5μg. IAA, increasing concentrations of kinetin also favor the elongation of germlings; the highest concentration tried (10 μg.%) induced the longest germlings obtained in these early experiments (Fig. 5). When adenine was superadded, the number of germlings induced by the combined action of IAA and kinetin is apparently not affected, but adenine completely inhibits the sharp elongation produced by 10 μg.% kinetin (Fig. 8). Perhaps in *Ulva* these morphogenetic determinants interact and have a limited specific action paralleling their activities on the tissues of higher plants.

At this point, we tried to substitute sea water media with synthetic mineral

Explanation of Plate I

Figures 1–8. Medium ASW III: forty-five days' growth. The new growth is represented by the lateral filaments budding from the old pieces. Figure 1: ASW III alone. Figure 2: + kinetin 2.5 μg.%.

Figures 3–5. Kinetin curve: basal medium = ASW III + indolacetic acid 5 μg.%; Figure 3: + kinetin 1 μg.%; Figure 4: + kinetin 5 μg.%; Figure 5: + kinetin 10 μg.%.

Figures 6–8. Kinetin curve: basal medium = ASW III + indolacetic acid 5 μg.% + adenine 3 mg.%; Figure 6: + kinetin 1 μg.%; Figure 7: + kinetin 5 μg.%; Figure 8: + kinetin 10 μg.%.

Figure 9. ASW 8 medium + adenine 3 mg.% + kinetin 20 μg.%. Same as Figure 15 but after 120 days' growth. Note islands of green, resistant cells interspersed in the bleached tissue of the blade.

Figures 10–20. ASW 8 medium: sixty days' growth. Figures 10–13. Indolacetic acid curve: basal medium = ASW 8 + kinetin 10 μg.%; Figure 10: No addition; Figure 11: + indolacetic acid 5 μg.%; Figure 12: + indolacetic acid 10 μg.%; Figure 13: + indolactic acid 20 μg.%.

Figures 14–15. ASW 8 medium + adenine 3 mg.%; Figure 14: No addition; Figure 15: + kinetin 20 μg.%.

Figures 16–20. Gibberellins curve: basal medium = ASW 8 + indolacetic acid 5 μg.% + kinetin 10 μg.%. Figure 16: No addition; Figure 17: + gibberellins 1 μg.%; Figure 18: + gibberellins 10 μg.%; Figure 19: + gibberellins 40 μg.%; Figure 20: + gibberellins 100 μg.%: note that all the filaments are bleached and that only the rhizoids of the disc of attachment are still green.

Figure 21. Same as Figure 18, but after 120 days' growth; note the many knobby islands of resistant green cells interspersed on the bleached filaments.

Enlargement of all figures 2 × natural size.

media (Provasoli *et al.*, 1957) or with other types of enriched sea water to which we added the most effective hormone combination (*i.e.*, IAA 5 μg.% and kinetin 10 μg.%). ASW 8 was far better than both ASW III and the synthetic media, and was used from then on.

In ASW 8, formation of germlings and germling elongation was again favored by the combination of kinetin and IAA. With kinetin constant at 10 μg.%, only rhizoids were formed when IAA was absent; 10 μg.% IAA elicited longer germlings than 5 μg.% IAA after 30 days growth, but at this concentration the tips of the germlings became brown in 60 days and the germlings were completely brown in 90 days (Fig. 12). The combination of kinetin 10 μg.% and IAA 5 μg.% produced healthy green germlings which kept on growing and the tips began to flatten, as happens normally in nature, at an earlier stage (Fig. 11); at higher concentrations (20 μg.%) IAA inhibited and only rhizoids were produced (Fig. 13).

Gibberellin, superimposed on the favorable concentrations of kinetin and IAA, induced a dramatic elongation. As with IAA, concentrations of gibberellin approaching the lethal induce a more rapid elongation: thus gibberellin at 100 μg.% produced the longest and thinnest filaments at 30 days growth, but growth stopped at this time and the filaments were totally bleached at 60 days (Fig. 20); only the rhizoids of the attachment disc remained green. Gibberellin at 10 μg.% elicited maximum elongation; concentrations between 10 and 40 μg.% neither inhibited nor reduced the number of green islands of cells left when the germlings bleached at the end of growth (Fig. 21). Gibberellin at 1 μg.% seemed to produce a definite response as compared with the control, but this may be due to a difference in inoculum (compare Fig. 17 with Fig. 11).

So far, the response of *Ulva* to morphogenetic substances had been to induce few or many, and shorter or longer, atypical solid filaments—a far cry from what happens in nature; still, a beginning of blade formation could be detected in the flattening at the tips of the germlings grown in kinetin 10 μg.% + IAA 5 μg.%. An elongated flat blade, probably composed of two layers of cells and similar to the one normally occurring in nature, was obtained by the addition of 20 μg.% kinetin to 3 mg.% adenine (Fig. 15). Adenine alone and lower concentrations of kinetin (2.5, 5, 10 μg.%) + adenine were completely ineffective; only rhizoids and lumpy growth around the inoculum appeared; the atypical elongated germlings were completely lacking (Fig. 14).

Discussion

Though the studies of *Ulva in* bacteria-free culture are just beginning, two unexpected findings emerge: 1) a sea weed under our experimental conditions requires exogenous hormones for normal morphogenesis; 2) the thalli, typical and atypical alike, reach only an extremely small size as compared with the natural one, then bleach, but only partially, leaving islands of green cells which, when transferred to new medium, can originate new germlings.

Thuret (1878) described only two morphological types of cells in *Ulva:* the oblong cells constituting the major portion of the thallus and the rhizoids which make up the disc of attachment. The rhizoids are formed by "tubular cells" originating in the basal part of the foliaceous thallus: these cells elongate, push their tips downward between the two cell layers of thallus, reach the substratum to which

the thallus is attached, and form a mat of filaments which anchors the thallus solidly.

Delf (1912) found that the tubular cells differ clearly from the other cells of *Ulva* in being multinucleate (they have 3–5 nuclei in the upper portion, few in the tubular portion and 2–5 nuclei in the rhizoidal portion). These observations were made on discs of *Ulva* growing on thalli of *Polysiphonia;* the material had been fixed in the early spring (*i.e.,* before the appearance of foliaceous thalli) yet the tubular cells were undoubtedly alive when fixed. Schiller (1907) believes that new germlings can originate from the rhizoids and Cotton (1910) and Delf (1912) postulate that the foliaceous part of the thallus of *Ulva* is annual while the disc of attachment is perennial.

Similarly, our experiments show that there are two types of cells: one which bleaches and dies easily, and a very resistant one. However, the resistant cells are located in two regions: 1) the disc of attachment, and 2) the erect elongated portion of the germling in which islands of cells remain green when the whole germling bleaches. Though both of these permanently green cells produce new germlings, they seem to have a different resistance to unfavorable conditions. At inhibitory concentrations of IAA (10 μg.%; Fig. 12) and gibberellin (100 μg.%; Fig. 20) no green islands appeared, all the cells of the erect part of the germling died, but the rhizoids remained green; at higher concentrations of IAA (20 μg.%; Fig. 13) the green islands of the inoculum did not produce new germlings but only a mat of rhizoids. It is most probable that the cells constituting the mat of the disc of attachment in our cultures are rhizoids, nonetheless we intend to test this hypothesis cytologically and see whether they are polynucleate. One would be tempted to consider that the cells of the green islands are also polynucleate because they are able to produce new germlings directly and without passing through the zoospore stage. However, they could also be morphologically identical with the oval cells which normally produce zoospores, but have a different potency. These cells do not appear only in the atypical germlings obtained in the laboratory; the original pieces of *Ulva,* from which our cultures derive, were small squares cut from the upper median part of the foliaceous thallus which is supposedly composed only of cells producing zoospores or gametes, yet not only zoospores were produced but a number of germlings originated directly from the piece of thallus which took the appearance, as noted, of a pincushion. Islands of permanently green cells in our cultures not only appeared in the atypical filamenteous germlings (Fig. 21), but also in the bleaching flat blade obtained with adenine + kinetin (Fig. 9). We can conclude then that another type of cell (different at least in its physiological potencies) exists among the oblong cells of the growing germlings and of the foliaceous thallus. These observations invite new studies on the morphology and potencies of the cells of *Ulva,* and on the localization and distribution in the various parts of the thallus of the various morphological and physiological types of cells. The resistant rhizoids of the attachment disc may prove the commonest and most valuable way of surviving winter and other hardships. The presence of other resistant cells in the foliaceous part of the thallus may be equally important ecologically in providing a more efficient way of spreading the species: pieces of thallus, fragmented by waves and transported by currents, can easily colonize distant sites far beyond the reach of the short-lived swimming zoospore stage.

Skoog and Miller (1957), in a penetrating review, conclude that regulation of

growth may depend more upon the quantitative interactions than upon the qualitative action of the single plant hormones. This contrasts with the previous ideas that there are specific organ-forming substances and that "determination" is an irreversible loss in the regenerative abilities of cells and tissues.

We have not yet explored separately the action of each morphogenetic substance in its active range, nor the effects of kinetin at higher concentrations nor all the various combinations of morphogenetic agents. It seems, at this stage, that production of more germlings and, especially, the elongation into atypical germlings result from the combined action of indolacetic acid and kinetin; adenine and indolacetic acid appear antagonistic.

The narrow effective range of IAA is puzzling. Judging from the elongation of the atypical filaments, kinetin and gibberellin are not toxic over a wide range, while indolacetic acid is effective only in a narrow range (1 μg.% IAA is barely active, 5 are optimal, and 10 μg.% induce rapid growth followed by rapid death). The formation of a flat thallus, so far, has been obtained by combining adenine with kinetin, but in this combination kinetin is inactive up to 20 μg.%, while in combination with indolacetic acid it elicits elongation of atypical germlings at 10 μg.% (Fig. 5). So far, only adenine + kinetin have given normal growth while growth of atypical germlings results from the combined action of kinetin and IAA.

Distinguishing between specific actions, interactions, and mixed actions of plant hormones is a complicated task in higher plants: isolated specialized tissues—an artificial situation—may be misleading for morphogenetic conclusions; mixed tissues represent different potencies, while organs are too highly specialized and reflect the interdependency of many distinct tissues.

If other algae respond to plant hormones as one may expect, they may become excellent experimental material. The Chlorophyceae, because of their closeness to the primitive land plants, may be the best choice: they abound in species representing practically all the early steps of increasing structural complexity—the simple filament; different types of heterotrichous filaments; complex branched filamentous thalli in which the prostrate and the erect system may be unequally developed; thalli with specialized oogamy; and foliaceous thalli. With algae, one can work with *whole organisms,* and not with parts of highly evolved organisms artificially avulsed from the whole, simply by selecting species in order of increasing morphological complexity. The activity of plant hormones on *Ulva* raises the question of precisely where in the algal line of evolution toward the land plants, plant hormones were first employed as morphogenetic regulators.

Earlier studies on the action of indolacetic on unicellular algae seem unconclusive or negative. Preliminary experiments, done in collaboration with J. J. Pintner and K. Gold, show that several fresh water and marine unicellular algae and even the colonial *Volvox globator* do not respond to indolacetic acid, kinetin and gibberellin: growth rate, final growth and morphology are unchanged within the concentration range effective for *Ulva.* It is not surprising that flagellates which are considered morphologically the primitive form from which the vegetal and animal tendencies of the algae have evolved, do not respond to morphogenetic hormones. Hormones are concerned with the balanced growth of a cellular organism—how can one expect to find visible changes in an organism which has no cellular parts? The lack of effect on *Volvox* supports the generally held idea that

this line is an evolutionary *cul-de-sac* and that *Volvox* is a colony of individuals. However, since the cell is the site of action of the hormones, "unicellular" algae may be the material of choice for studying the mode of action of plant hormones at the cellular level, but then, we need powerful specific antagonists to plant hormones.

It has been fortunate that plant hormones under our experimental conditions are indispensable for normal morphogenesis of *Ulva*. Quite likely this will hold for other media but, if it were not so, some nutritional factors upset the normal morphological development; the study of their role in morphogenesis could then allow a deeper insight into the action of plant hormones. ASW 8 allows better growth and the action of plant hormones is more evident in ASW 8 than in ASW III. The main difference between the two media is the presence in ASW III of an aqueous liver extract and soil extract, both of which introduce purines. Some purines, as adenine and kinetin, are important morphogenetic agents for *Ulva,* but it is conceivable that other purines may interfere with the normal processes of growth.

Definitive results can be obtained only by substituting for sea water a chemically defined medium to eliminate the unknown organic constituents of sea water.

The *Ulva* data suggest that these plant hormones may be as ecologically important for other sea weeds as vitamins are for phytoplanktonts. The auxins and gibberellins are microbial products (see Brian's review, 1957) and the unknown natural purines, which act like kinetin, may also be significantly contributed in natural waters by microbial action. It is possible therefore that the coastal zone, because of the land drainage which favors microbial growth, may never be so poor in plant hormones as to limit sea weed growth severely, but fluctuation in their level may control speed of growth and size of crop. This may be of economic importance to nations, like Japan and Ireland, which farm and use sea weeds extensively. To resolve these issues, not only are extensive pure culture studies needed but also convenient sensitive methods for assaying plant hormones in sea water.

Summary

1. Bacteria-free *Ulva lactuca,* in sea water media enriched with vitamins, grows as atypical, short, filamentous germlings which do not develop into a foliaceous thallus. These filaments reach a few millimeters, then bleach, leaving a few islands of intensely green cells which, upon transfer to new media, produce new germlings.

2. The initiation of new germlings from these green islands and the length of the atypical filaments are increased by the combination of kinetin + indolacetic acid; adenine and indolacetic acid seem antagonistic. Conspicuous elongation of the filaments is promoted by the addition of gibberellins to the kinetin-indolacetic acid combination.

3. A normal flat blade was obtained so far only with adenine + kinetin.

4. The responses depend both on the interaction and concentrations of these morphogenetic agents: indolacetic acid is effective only in a very narrow range of concentrations and a blade is produced only with relatively high concentrations of kinetin.

5. The rhizoids of *Ulva* and the cells of the green islands can produce directly new germlings and are far more resistant to unfavorable conditions than the other

cells of the thallus which can originate zoospores or gametes. The morphogenetic and ecological significance of the resistant cells is discussed.

6. These responses of a relatively simply organized sea weed to plant hormones link even more tightly the green algae to the higher land plants.

7. The variety of evolutionary steps toward increased morphological complexity in the algae suggests that whole organisms, because of their relative morphological simplicity, may be valuable experimental material for studying the mode of action of plant hormones.

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